Regulators of Spermatogenesis in *Drosophila*

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Abstract

Sperm cells are some of the most highly specialized cells in animals. Their unique function, to carry male genetic information, and morphology are highly conserved across the animal world. Spermatogenesis, the production of sperm, is strikingly similar in *Drosophila melanogaster* and mammals, making *Drosophila* an excellent model. Individualization of the spermatids is required to finalize the development of the 64 spermatozoa. The cell communication pathway JAK-STAT (Janus Kinase, Signal Transducer and Activator of Transcription) is required to induce individualization. Prior to individualization, JAK-STAT signaling is activated in the somatic cyst cells by the spermatids. The two somatic cyst cells surrounding the spermatids send a signal to the spermatids to begin individualization. To study which genes might have a role in individualization, a candidate gene list was created from genes that were differentially expressed after JAK-STAT signaling was impaired in somatic cells. Candidate genes were knocked down or overexpressed specifically in the germline (VasaGAL) or the somatic cells (EyaGAL) using the GAL4-UAS system. Many genes showing statistically significant differences compared to the control were metalloproteases, where overexpression of a metalloprotease inhibitor in somatic cyst cells might be having an effect on individualization. Genes expressed early in individualization responsible for cell polarity produced underdeveloped testes when knocked down.

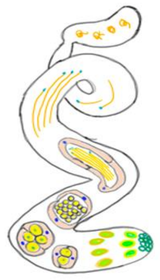

Figure 1: Spermatogenesis in *Drosophila*. Sperm is produced through the process of spermatogenesis. Germline stem cells divide mitotically then meiotically from the hub of the testis until 64 spermatocytes are surrounded by two somatic cyst cells. The spermatocytes are then elongated and individualized.

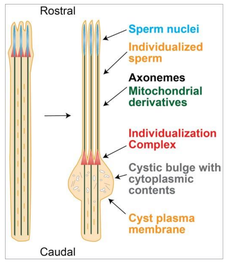

Figure 2: Individualization of Spermatids. Individualization is the last step of spermatogenesis. 64 investment cones form around the 64 nuclei of the nuclei bundle to form an individualization complex (IC). The IC then takes excess cytoplasm and waste as it moves away from nuclei to individualize and compact the spermatids.

Experimental Design and Methods

Candidate Gene Selection

To identify candidate genes that are involved in individualization, RNA profiling was performed on testes from flies with impairment of the JAK-STAT pathway in somatic cyst cells and compared with wild-type controls (Figure 3). RNA sequencing of these lines was used to create a candidate gene list with 336 significantly down-regulated genes. Using four ontology terms – signal, membrane, reproduction, and stat binding – the genes were categorized (Figure 4), and genes with overlap in multiple categories were prioritized for functional study (i.e. MMP2 and IDGF2).

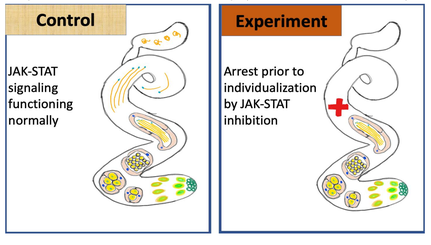

Figure 3: Candidate Gene Selection Experiment. JAK-STAT was impaired in cyst cells, arresting developmental prior to individualization. Extracted RNA was profiled to identify differential expressed genes.

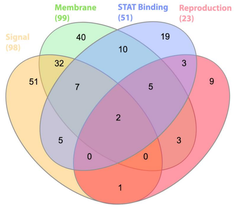

Figure 4: Gene Ontology Venn Diagram. Categorization of differentially expressed candidate genes by four ontology terms: signal, membrane, stat binding, and reproduction.

Genetic Manipulation of Candidates

The GAL4-UAS system was used to knockdown or overexpress candidate genes. The system involves a driver line, with a tissue specific promotor, and a responder line, having a UAS binding site. When these lines are crossed, the tissue specific promotor will produce GAL4 and bind to the UAS site in the progeny. This binding either drives the expression of a gene or, if using RNAi, will knockdown the gene (Figure 5). This experiment utilized RNAi to knockdown genes, except for Timp, where the gene was overexpressed.

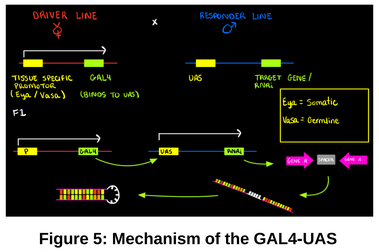

Figure 5: Mechanism of the GAL4-UAS System. The GAL4-UAS system utilizes driver and responder lines. When crossed, a tissue specific promotor from the driver line produces GAL4, which binds to the UAS binding site in the responder. This binding either causes expression of a target gene, or knockdown if using RNAi.

Analysis of Individualization Phenotypes

Virgin females from driver lines (VasaGAL and EyaGAL) were crossed with males from candidate gene responder lines and reared at room temperature. Male progeny were collected from the crosses and aged at 27 °C for 3-5 days initially, and then later 5-7 days. After aging, the male testes were dissected, fixed, and stained with DAPI and Phalloidin.

Results & Discussion

Genes Knocked Down in the Germline Led to Impairment of Individualization

A minimum of 25 testes were dissected from each knockdown and scored for five criteria. The scores were averaged, and the data was analyzed with Student T-tests using the total IC numbers and nuclei bundle to IC ratios. Categories within a genotype that demonstrated a statistically significant difference from the control are marked with an asterisk in the following figure.

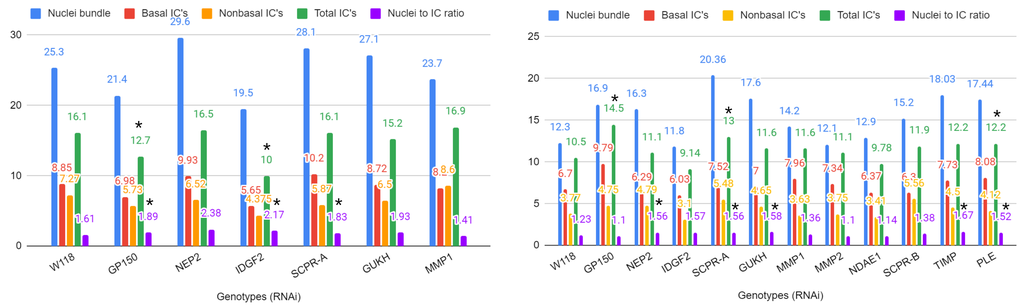

Figure 6: Results for Control (W118) and Knockdown Genotypes for VasaGAL Driver at 3-5 Days (Left) and 5-7 Days (Right). Candidate genes lines for VasaGAL driver were sored using five criteria. Total IC and nuclei to IC ratio were compared to control using a Student's T-test. Values with a statistically significant difference from the control had a p value <0.05 and are indicated by an asterisk.

RNAi knockdown of Nep2 in the germline stem cells (VasaGAL) showed a statistically significant difference in the nuclei bundles to IC ratio. DAPI staining of Nep2-RNAi and W118 control (top left and bottom left), shows the increased number of nuclei bundles in the Nep2-RNAi testis compared to the W118 control (Figure 7). Gp150-RNAi and Idgf2-RNAi showed statistically significant differences from the control in both the average total IC count and the nuclei to IC ratio. The Phalloidin staining (indicated by arrows) shows the reduced number of IC's in the knockdowns compared to the control (Figure 7).

Figure 7: Comparison of VasaGAL Driver Knockdown Genes – Nep2, Gp150, and Idgf2 – to VasaGal4;W118 (control). Nep2-RNAi had a statistically significant difference from the control in the nuclei to IC ratio, seen by the increased number of nuclei bundles in the Dapi staining (white boxes). Gp150-RNAi and Idgf2-RNAi had statistically significant differences from the control in both the total IC count and the nuclei to IC ratio, depicted by the smaller amount of IC's in the Phalloidin staining for the knockdowns compared to control (white arrows).

Genes Knocked Down in the Somatic Cyst Cells Led to Impairment of Individualization

A minimum of 25 testes were dissected from each knockdown/overexpression and scored for five criteria. The scores were averaged, and the data was analyzed with Student T-tests using the total IC counts and nuclei bundle to IC ratios. Categories within a genotype that demonstrated a statistically significant difference from the control are marked with an asterisk in the figure below.

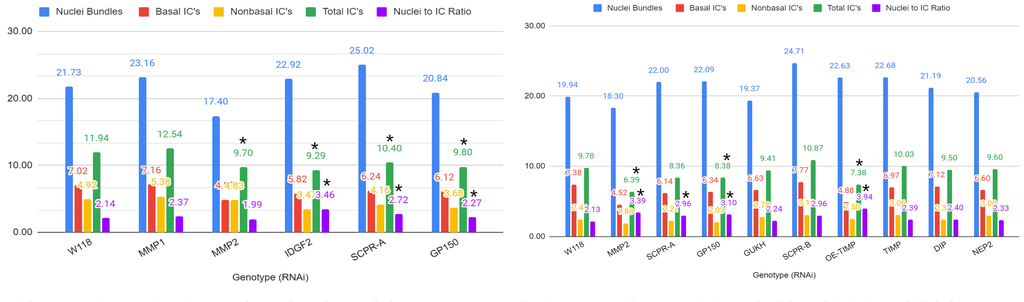

Figure 8: Results for Control (W118) and Knockdown Genotypes for EyaGAL Driver at 3-5 Days (Left) and 5-7 Days (Right). Candidate genes lines for EyaGAL driver were sored using five criteria. Total IC and nuclei to IC ratio were compared to control using a Student's T-test. Values with a statistically significant difference from the control had a p value <0.05 and are indicated by an asterisk.

Knockdown of both Gp150 and Idgf2 eventually showed significant difference from the control in the average total IC count and the nuclei to IC ratio tests. Idgf2 and Gp150 knockdowns produced actin staining that does not correspond to ICs.

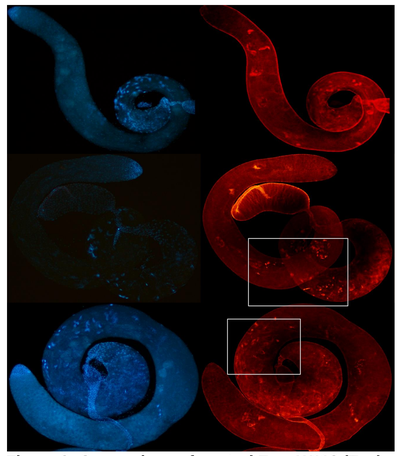

Figure 9: Comparison of control Eya; W118 (Top), Eya; Idgf2-RNAi (Middle), and Eya; Gp150-RNAi with both Dapi and Phalloidin Staining. Both knockdowns exhibited a reduction in number of total IC's and an increase in the nuclei to IC ratio when compared to the control. Both knockdowns also contain irregular actin staining that is not as severe in the control (white box).

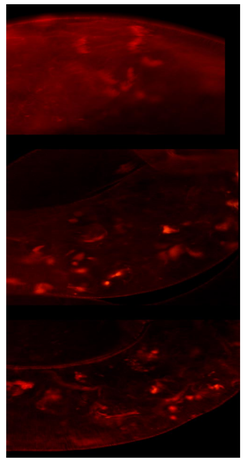

Figure 10: Comparison of Eya; W118 (Top), Eya; Idgf2-RNAi (Middle), and Gp150-RNAi with Phalloidin Staining. Magnified images of the phenotypes show the contrast between the IC's of the control and the IC's of the knocked-down genes

Somatic Cell Knockdown of I(2)gl and dlg1 Led to Underdeveloped Testes

These cells polarity genes are expressed early in spermatogenesis and are crucial to proper development of the testis. These genes are involved in maintaining a proper microenvironment for cyst formation. Previous studies have shown that knockdown of dlg1 and I(2)gl in somatic cyst stem cells has led to much smaller testes with impaired differentiation.

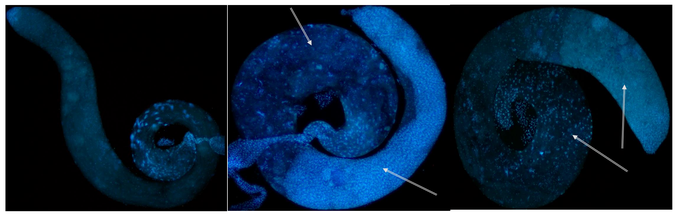

Figure 11: Eya; W118 (Left)-RNAi, dlg1 (Middle)-RNAi, and I(2)gl(Right)-RNAi Dapi Staining Comparison. The RNAi knockdowns of genes dlg1 and I(2)gl produced testes that were smaller in comparison to the control and also lacked elongated nuclei bundles. Lack of elongated nuclei near the base of the testis and the presence of highly concentrated Dapi staining near the apical tip suggests germline cells are not differentiating properly (arrows).

Conclusions

- Knockdown of a metalloprotease and overexpression of a metalloprotease inhibitor in somatic cyst cells and germline stem cells caused impaired spermatogenesis/individualization
- A transmembrane glycoprotein gene, as well as genes known to have functions in the extracellular matrix, impaired individualization when knocked down in the somatic cyst cells and germline stem cells
- Knockdown of dlg1 and I(2)gl with EyaGAL driver impaired spermatogenesis prior to individualization and led to underdeveloped testes
- Candidate genes from this study that demonstrated a statistically significant difference from the control appear to have a role in the signaling by somatic cyst cells to initiate individualization

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